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This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

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Additional inventors are being named on the _____ separately numbered sheets attached hereto					
TITLE OF THE INVENTION (500 characters max)					
Biotherapeutics, Diagnostics and Research Reagents					
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ENCLOSED APPLICATION PARTS (check all that apply)					
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<input checked="" type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27.					
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[Page 1 of 2]

Respectfully submitted,

SIGNATURE

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Date January 5, 2004

REGISTRATION NO. 46,731

(if appropriate)

Docket Number: QT-0002-P

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☒ Applicant claims small entity status. See 37 CFR 1.27

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Filing Date	January 5, 2004
First Named Inventor	Simon Delagrave
Examiner Name	na
Art Unit	na
Attorney Docket No.	QT-0002-P

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Fee Code	Fee (\$)	Fee Code	Fee (\$)		
1001	770	2001	385	Utility filing fee	
1002	340	2002	170	Design filing fee	
1003	530	2003	265	Plant filing fee	
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1005	160	2005	80	Provisional filing fee	80.00
SUBTOTAL (1)					(\$ 80.00)

2. EXTRA CLAIM FEES FOR UTILITY AND REISSUE

Total Claims -20** = X =

Independent Claims -3** = X =

Multiple Dependent =

Large Entity		Small Entity		Fee Description	Fee Paid
Fee Code	Fee (\$)	Fee Code	Fee (\$)		
1202	18	2202	9	Claims in excess of 20	
1201	86	2201	43	Independent claims in excess of 3	
1203	290	2203	145	Multiple dependent claim, if not paid	
1204	86	2204	43	** Reissue independent claims over original patent	
1205	18	2205	9	** Reissue claims in excess of 20 and over original patent	
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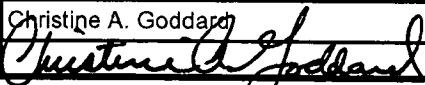
Fee Code	Fee (\$)	Fee Code	Fee (\$)	Fee Description	Fee Paid
1051	130	2051	65	Surcharge - late filing fee or oath	
1052	50	2052	25	Surcharge - late provisional filing fee or cover sheet	
1053	130	1053	130	Non-English specification	
1812	2,520	1812	2,520	For filing a request for <i>ex parte</i> reexamination	
1804	920*	1804	920*	Requesting publication of SIR prior to Examiner action	
1805	1,840*	1805	1,840*	Requesting publication of SIR after Examiner action	
1251	110	2251	55	Extension for reply within first month	
1252	420	2252	210	Extension for reply within second month	
1253	950	2253	475	Extension for reply within third month	
1254	1,480	2254	740	Extension for reply within fourth month	
1255	2,010	2255	1,005	Extension for reply within fifth month	
1401	330	2401	165	Notice of Appeal	
1402	330	2402	165	Filing a brief in support of an appeal	
1403	290	2403	145	Request for oral hearing	
1451	1,510	1451	1,510	Petition to institute a public use proceeding	
1452	110	2452	55	Petition to revive - unavoidable	
1453	1,330	2453	665	Petition to revive - unintentional	
1501	1,330	2501	665	Utility issue fee (or reissue)	
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1806	180	1806	180	Submission of Information Disclosure Stmt	
8021	40	8021	40	Recording each patent assignment per property (times number of properties)	
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1810	770	2810	385	For each additional invention to be examined (37 CFR 1.129(b))	
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SUBTOTAL (3) (\$)**SUBMITTED BY**

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Name (Print/Type)	Christine A. Goddard	Registration No. (Attorney/Agent)	46,731	Telephone	215-665-2191
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BIOTHERAPEUTICS, DIAGNOSTICS AND RESEARCH REAGENTS

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FIELD OF THE INVENTION

The present invention relates to polypeptides that are useful in methods of detecting pathogens as well as diagnosing and treating diseases. The polypeptides contain a PDZ domain capable of binding with a target produced by a pathogen or disease state. *In vitro* evolution processes can be used to prepare the polypeptides of the invention.

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BACKGROUND OF THE INVENTION

Availability of proteins that specifically bind or interact with target proteins or other molecules has for some time been of importance in biology and medicine. For example, medical diagnosis has been revolutionized by assays using high-affinity proteins, mainly antibodies, that bind to disease markers. High-affinity antibodies to disease-causing agents are of increasing importance in medical therapeutics. In biological research, high affinity proteins, also mainly antibodies, have found use in the purification of rare proteins, in the localization of proteins or other antigens in cells such as by immuno-histochemical techniques, and in countless other applications. High-affinity proteins are likely to assume increasing research importance in the future. For example, the emerging field of proteomics seeks to understand the patterns of expression and interaction of a substantial fraction of the proteins encoded in a cell's genome.

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However, existing methods of providing binding proteins or polypeptides that bind with affinity and specificity to selected targets, especially to large numbers of selected targets, has been and continues to be difficult and expensive. The predominant method used today is to raise antibodies, either monoclonal or polyclonal, against a target molecule. Although well known and widely used, this strategy has several limitations and disadvantages. First, to generate, or "raise", an antibody against a target requires either a sufficient amount of the purified target itself or a chemically synthesized fragment of the target. Second, raising an antibody normally requires the use of living animals, and due to species incompatibilities, it is not always possible to raise a specific antibody against a

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particular target, much less against large numbers of targets, such as a significant fraction of the proteins in an organism. Third, isolation and production of antibodies are expensive, time-consuming and unpredictable processes. Fourth, antibodies cannot be expressed via recombinant hosts without significant investment of time and money because the antigen-binding regions of the antibody heavy and light chains must be cloned, sequenced, and then simultaneously expressed. Finally, antibodies usually do not fold properly in the reductive cell environment, and therefore are not useful to target intracellular molecules involved in disease. Such limitations and disadvantages constitute a significant barrier to the rapid identification, diagnosis and treatment of infectious diseases such as AIDS, SARS, West Nile virus, and anthrax, or of non-infectious diseases such as cancer.

An alternative method relies on “directed evolution” to alter the binding specificity of naturally-occurring proteins that are known to bind to determined targets. In this method, a known gene is randomly mutated by a chemical or biotechnological mutagenesis technique, for example, by PCR-based mutagenesis. Then a library of the resulting protein variants is screened for variants having affinity to a new target, for example, by phage display. In this way, several proteins have been “evolved” in the laboratory to create protein variants having useful new specificities (e.g., Xu et al., 2002, Chem Biol, 9: 933).

A further alternative is to create novel binding proteins *de novo* through directed evolution. However, proteins having no natural counterparts, e.g., iMabs from Catchmabs BV or as described by (Keefe et al., 2001, Nature, 410: 715-8), have a significant drawback in that they are likely to be recognized as foreign by the human immune system, thereby impeding their use as therapeutics. For the same reason, natural proteins of non-human origin engineered to bind target polypeptides (e.g., Ronnmark et al., 2002, Eur J Biochem, 269: 2647-55.; Zeytun et al., 2003, Nat Biotechnol, 21: 1473-9.) are unlikely to be useful as therapeutics or diagnostics.

Thus, the choice of binding protein to be modified via directed evolution will strongly influence the utility of the evolved binding proteins. PDZ domains constitute an example of a family of binding proteins which can be used to create novel research reagents, diagnostic reagents or therapeutics having many advantages over existing binding proteins. Such advantages include ease and speed of isolation using *in vitro* methods, low cost of production using non-mammalian host cells, potential utility as intracellular biotherapeutics due to their natural propensity to function in the cytoplasm, and lack of immunogenicity.

PDZ domains are relatively well understood and of great potential utility. They participate in signal transduction pathways by mediating protein complex formation and are also involved in targeting of proteins to various locations within the cell. In metazoan genomes, including the human genome, PDZ domains are among the most common protein sequence modules. Recent reviews on PDZ domains include refs. (Hung et al., 2002, J Biol Chem, 277: 5699-702) and (Fan et al., 2002, Neurosignals, 11: 315-21). Many PDZ domains are stable and expressed to high levels in recombinant bacterial hosts, which has facilitated their extensive biophysical characterization (e.g., Morais Cabral et al., 1996, Nature, 382: 649-52.; Cohen et al., 1998, J Cell Biol, 142: 129-38.; Daniels et al., 1998, Nat Struct Biol, 5: 317-25.; Im et al., 2003, J Biol Chem, 278: 8501-7). PDZ domains have been described as potential therapeutics, for example to treat cancer by interfering with Myc protein function. See for example, (Junqueira et al., 2003, Oncogene, 22: 2772-81) and US patent application 20030119716. Other PDZ patent applications expand the utility of PDZ domains by describing engineered PDZ domain fusions, or chimeras, with other proteins (e.g., US Patent Application Pub. Nos. 20010044135, 20020037999, 20020160424). PDZ domains can also be used to identify drug candidates in high-throughput screens (Ferrer et al., 2002, Anal Biochem, 301: 207-16; Hamilton et al., 2003, Protein Sci, 12: 458-67).

Some progress has been made in studying and modifying the binding specificity of PDZ domains. Schneider et al., 1999, Nature Biotechnology 17:170-175 and (Junqueira et al., 2003, Oncogene, 22: 2772-81) both describe how the binding specificity of a naturally-occurring PDZ domain can be altered using directed evolution methods. Phage display may be used to determine the specificity of a given PDZ domain (see, e.g., Fuh et al., 2000, J. Biol. Chem. 275:21486-91). In this work, Fuh and colleagues selected phage-displayed random C-terminal peptide sequences capable of binding to an immobilized PDZ domain. However, this approach is not intended to, and cannot alter the specificity of a given PDZ domain. In contrast, Skelton et al. (2003, J. Biol. Chem., 278: 7645-54), propose the use of phage display to alter PDZ domain specificity, but do not demonstrate it. Phage display is believed to provide greater control over the conditions of the binding interactions, including affinity and specificity, than is afforded by two-hybrid selections which are notoriously artifact-prone.

Alternatively, PDZ domains with altered binding specificity may be designed by computational methods, as shown by (Reina et al., 2002, Nat Struct Biol, 9: 621-7) and US Patent Application Pub. No. 20030059827. These computational methods seem to offer

several apparent benefits, such as reduced cost and time by avoiding experimental effort, and scalability for determining binding proteins to multiple targets. On the other hand, these methods have certain notable drawbacks such as the well-known extreme difficulty of predicting binding affinities of designed protein structures, yielding candidate binding proteins of unreliable affinity and specificity. Also, once structures have been designed *in silico*, the corresponding proteins must still be prepared in the laboratory. The effort required to construct the candidate gene variants is similar to the effort required to prepare a library of mutant genes, and once such a library is constructed, it can be screened multiple times with diverse targets whereas new variants must be designed and synthesized for each new target. Finally, design of variant binding proteins and optimization of their binding affinity is extremely difficult without the availability of detailed information on their atomic structure, while directed evolution has no such need. The acquisition of this type structural data is costly and slow, often requiring months of work.

In summary, polypeptides capable of binding to specific targets, especially to natural peptide sequences, are useful in biology and medicine, and are expected to be of increasing utility in the future. But the current art offers no methods sufficiently efficient and economical to meet demands for large numbers of versatile binding proteins. Existing methods are time consuming, often costly, and may have additional drawbacks. Therefore, inexpensive and efficient methods for providing diverse binding proteins capable of functioning as affinity reagents and/or therapeutics are needed.

SUMMARY OF THE INVENTION

The present invention provides a polypeptide comprising a PDZ domain, wherein the PDZ domain binds to a target produced by a pathogen or disease state. In some embodiments, the pathogen is viral, fungal, or bacterial. In further embodiments, the disease state is cancer. In some embodiments, the pathogen is *Bacillus anthracis*.

The present invention further provides a polynucleotide encoding a polypeptide of the invention, a vector containing the polynucleotide, or host cell containing the polynucleotide. Also provided is an antibody that binds to a polypeptide of the invention.

The present invention further provides a method of detecting the presence of a pathogen or disease in a patient comprising administering a polypeptide of the invention to the patient; and detecting binding of the polypeptide in the patient.

The present invention further provides a method of detecting the presence of a pathogen or disease in a sample comprising contacting a polypeptide of the invention with the sample; and detecting binding of the polypeptide to the sample.

5 The present invention further provides a method of preparing a polypeptide comprising a PDZ domain, wherein the PDZ domain binds to a target produced by a pathogen or disease state, comprising: creating a library of polypeptides from one or more parent polypeptides comprising a PDZ domain; and identifying one or more polypeptides from the library having binding affinity for the target.

10 The present invention further provides a polypeptide comprising a PDZ domain, wherein the PDZ domain binds to a target, wherein the polypeptide is prepared by: creating a library of polypeptides from a parent polypeptide comprising a PDZ domain having SEQ ID NO: 2; and identifying the polypeptide having binding affinity for the target from the library.

15 The present invention further provides a polypeptide comprising a PDZ domain, wherein the PDZ domain binds to a target and wherein the polypeptide is prepared by recursive ensemble mutagenesis.

The present invention further provides a library of polypeptides prepared from a parent polypeptide comprising a PDZ domain, wherein the parent polypeptide comprises SEQ ID NO: 2.

20 The present invention further provides a method of treating a disease associated with a pathogen, comprising administering to a patient infected with or likely to become infected with the pathogen a therapeutically effective amount of a polypeptide comprising a PDZ domain capable of binding to a target associated with the pathogen. In some embodiments, the pathogen is *Bacillus anthracis*, *Clostridium botulinum*, or *Clostridium tetani*.

25 The present invention further provides a method of preparing a polypeptide comprising a PDZ domain, wherein the PDZ domain binds to a polypeptide target produced by a pathogen or disease state, comprising: forming a library of polypeptides from one or more parent polypeptides comprising a PDZ domain; selecting a first polypeptide from the library, where the first polypeptide has binding affinity to an intermediate target having 20% to 80% sequence identity in the last 5 amino acids with the last 5 amino acids of the target;
30 creating a further library of polypeptides from the first polypeptide; and repeating the prior two steps until a polypeptide that binds with the target is identified.

The present invention further provides a method of purifying a protein comprising contacting the protein with an immobilized polypeptide comprising a PDZ domain, wherein the immobilized polypeptide has binding affinity for the protein.

5 BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Multiple sequence alignment of proteins found in a BLAST search of the “nr” protein database using hCASK PDZ domain as a query. Identities are shown as dots. Upper panel of figure shows relevant sequences for residues M501, I503 and L505. Lower panel shows relevant sequences for residues Q553, L556 and R557. Underlined numbers on left-
10 hand side of the figure correspond to NCBI GI numbers.

DESCRIPTION OF EMBODIMENTS OF THE INVENTION

The present invention provides, *inter alia*, a polypeptide comprising a PDZ domain capable of binding to a preselected target, and methods of using and preparing the
15 polypeptide. In some embodiments, the PDZ domain binds to a target produced by a pathogen or disease state. Example targets include naturally occurring proteins, peptides, or other molecules associated with, such as produced directly by, a pathogen or disease state, including non-infections disease states such as cancer and cardiopulmonary dysfunction.

20 Definitions

As used herein, “polypeptides” or “proteins” are polymers of amino acids having, for example, from 2 to about 1000 or more amino acid residues. Any naturally occurring or synthetic amino acid can form the polypeptide. Polypeptides can also include modifications such as glycosylations and other moieties. In some embodiments, polypeptides of the
25 invention have the ability to selectively bind to target polypeptides largely based on amino acid sequence of the target. These polypeptides can contain a binding domain such as a PDZ domain. In further embodiments, polypeptides can contain additional functional regions such as a “reporter group” and/or an “effector domain.”

As used herein, “PDZ domain” refers to a protein module capable of binding to a
30 target protein by recognition of the target’s C-terminal or N-terminal amino acid sequence. PDZ domains are typically 85-95 amino acids in length and are found naturally in a variety of organisms ranging from bacteria to humans. An example PDZ domain is the PDZ domain of hCASK having the sequence SEQ ID NO: 2. Other PDZ domains, according to the

invention, have homology to the PDZ domain of SEQ ID NO: 2, such as at least about 50 % identity using BLAST (default parameters). The name PDZ is derived from: PSD-95 (Cho et al., Neuron 9:929-942, 1992), Dlg-A (Woods and Bryant, Cell 66:451-464, 1991) and ZO-1 (Itoh et al., J. Cell. Biol. 121:491-502, 1993), each of which contains three such domains.

5 PDZ domains have also been called GLGF repeats or DHRs and are identified in a variety of proteins (Ponting and Phillips, Trends Biochem. Sci. 20:102-103, 1995). A PDZ domain of PTPL1 has been shown to interact with the C-terminal tail of the membrane receptor Fas (Sato et al., 1995) and PDZ domains of PSD-95 bind to the C-terminals of the NMDA-receptor and Shaker-type K⁺ channels (Kim et al., Nature 378:85-88, 1995; Kornau et al.,

10 Science 269:1737-1740, 1995). The crystal structures of different PDZ domains have been published (e.g., Doyle et al., Cell .85:1067-1076, 1996; Morais Cabral et al., Nature 382:649-652, 1996). The PDZ domain of human CASK/LIN-2, also called hCASK, is well studied: its substrate specificity has been investigated (Cohen et al., 1998, J Cell Biol, 142: 129-38.) and its crystal structure determined (Daniels et al., 1998, Nat Struct Biol, 5: 317-25.).

15 skilled in the art can readily recognize a PDZ domain.

PDZ domains can also be changed ("evolved") by an *in vitro* evolution process to generate a different PDZ domain having a particular desired function that is different from the original function. The "evolved" PDZ domain can be evolved from any parent PDZ domain to change affinity to or specificity for a preselected target. In some embodiments, the

20 PDZ domain is evolved from the hCASK PDZ domain.

A "reporter group", as used herein, is defined as a molecular moiety that is readily detected, directly or indirectly, and is attached covalently to a polypeptide, such as a polypeptide containing a PDZ domain. Examples of reporter groups include polynucleotides that are readily detected, for example, by polymerase chain reaction (PCR); biotin which is

25 readily detected with streptavidin conjugated to horseradish peroxidase; fluorescent proteins such as the Green Fluorescent Protein (GFP), which is detected by fluorescence spectroscopy; epitope tags such as the influenza hemagglutinin peptide HA epitope corresponding to the amino acid sequence YPYDVPDYA, detected with antibodies binding specifically to this epitope; dual function epitope/enzyme tags such as GST (glutathione S-

30 transferase), which can be detected indirectly using an antibody specific to this protein, or directly using a colorimetric assay measuring enzymatic GST activity; enzymes such as alkaline phosphatase, which can be detected using chemiluminescence. Reporter groups also include radioactive isotopes and imaging agents, such as chelated heavy metals, which can be

used for *in vivo* diagnostics and imaging. Numerous other examples of molecular entities which can be used as reporter groups are known in the art.

An “effector domain”, as used herein, is defined as a protein domain, or other molecular moiety, which adds a function other than detection to another protein domain, such as a PDZ domain, to which it is covalently attached. An effector domain can be the Fc domain of immunoglobulins, which mediates functions of the immune system such as opsonization, phagocytosis and activation of complement. Other effector domains include toxins such as cholera toxin, which can be used to kill cells recognized by the polypeptide to which the effector domain is attached. Other toxins can include, for example, botulin toxin, diphtheria toxin, anthrax toxin, ricin, *Clostridium difficile* toxin, and the like. Other examples of effector domains include protein transduction domains which enable proteins to which they are attached to cross the cell membrane and to locate in the cytoplasm of mammalian cells, as described, for example, in Wadia and Dowdy, 2002, Curr Op Biotechnol, 13:52-6 and references therein, in which short sequences such as the Tat protein’s transduction domain (YGRKKRRQRRR, single letter amino acid code) and other arginine-rich basic peptides are described. Another example effector domain is serum albumin. Yet other examples of effector domains include RNA molecules which can be used to mediate selective inactivation of gene expression via RNA interference (RNAi); chemotherapeutic agents such as bleomycin, which can be used to kill cancer cells; radioactive isotopes which can also be used to kill cancer cells; and the like. More than one effector domain can be linked to a single PDZ domain in a poly peptide of the invention.

The term “peptide” refers to a compound of 2 to about 50 subunit amino acids, amino acid analogs, or peptidomimetics. The subunits can be linked by peptide bonds. In other embodiments, the subunit can be linked by other bonds, e.g. ester, ether, etc. As used herein the term “amino acid” refers to either natural and/or unnatural or synthetic amino acids, including glycine and both the D or L optical isomers, and amino acid analogs and peptidomimetics. A peptide of about three or more amino acids is commonly called an oligopeptide if the peptide chain is short (e.g., under about 40 amino acids). If the peptide chain is long (e.g., more than about 50 amino acids), the peptide is commonly called a polypeptide or a protein.

As used herein, the term “*in vitro* evolution”, or “directed evolution” refers to a method of generating new polypeptides (e.g., a “library” of polypeptides) by accelerating mutation rates and/or recombination events of polynucleotides encoding parent polypeptides

under *in vitro* conditions and screening or selecting the resulting new polypeptides. The process of directed evolution has been described in detail (Joo et al., Chem. Biol., 1999, 6, 699-706; Joo et al., Nature, 1999, 399, 670-673; Miyazaki et al., J. Mol. Evol., 1999, 49, 716-720; Chen et al., Proc. Natl. Acad. Sci. USA, 1993, 90, 5618-5622; Chen et al.,
5 Biotechnology, 1991, 9, 1073-1077; You et al., Protein Eng, 1996, 9, 77-83; each of which is incorporated herein by reference in its entirety). In general, the method involves the steps of 1) creating a population of mutant genes; 2) screening this population for individual genes which have a desired property such as coding for a protein with improved binding affinity; and repeating these two steps, if necessary, until a desired improvement is achieved. Many
10 methods to introduce mutations exist and are described in the literature (Leung et al., Technique, 1989, 1, 11-15; Delagrave et al., Protein Eng., 1993, 6, 327-331; each of which is incorporated herein by reference in its entirety). Similarly, there are many ways to screen or select mutants for a desired property (Smith, *Science*, 1985, 228, 1315; Hanes & Pluckthun, Proc. Natl. Acad. Sci. USA, 1997, 94, 4937; Xu et al., Chem. Biol, 2002, 9, 933; Joo et al.,
15 Chem. Biol., 1999, 6, 699-706; Joo et al., Nature, 1999, 399, 670-673; Miyazaki et al., J. Mol. Evol., 1999, 49, 716-720; Chen et al., Proc. Natl. Acad. Sci. USA, 1993, 90, 5618-5622; Chen et al., Biotechnology, 1991, 9, 1073-1077; You et al., Protein Eng, 1996, 9, 77-83; Marrs et al., Curr. Opin. Microbiol., 1999, 2, 241-245; and U.S. Pat. No. 5,914,245).

As used herein, the term “parent polypeptide” describes a polypeptide which is a
20 starting component of an *in vitro* evolution process. “Parent polypeptide” distinguishes the starting polypeptides from evolved forms of the polypeptides (“evolved polypeptides”). For example, a “parent PDZ domain” refers to a PDZ domain that is used as a starting point for generating different (or evolved) PDZ domains by *in vitro* evolution. Likewise, an “evolved PDZ domain” describes a PDZ domain that is the product of an *in vitro* evolution process.

25 As used herein, the term “parent polynucleotide” describes a polynucleotide which is a starting component of an *in vitro* evolution process. “Parent polynucleotide” distinguishes the starting polynucleotides from evolved forms of the polynucleotides (“evolved polynucleotides”). For example, a “parent PDZ polynucleotide” refers to a polynucleotide encoding a PDZ domain that is used as a starting point for generating different (or evolved)
30 PDZ domains by *in vitro* evolution. Likewise, an “evolved PDZ polynucleotide” describes a polynucleotide encoding a PDZ domain which is the product of an *in vitro* evolution process.

As used herein, “library” refers to a collection of two or more different polypeptides or polynucleotides. The collection of polypeptides or polynucleotides of a library can be

prepared by any of numerous methods including error-prone PCR, recursive ensemble mutagenesis, and other mutagenesis methods such as gene shuffling and the like.

As used herein, a “target” refers to any molecular entity to which a further molecular entity binds. In some embodiments, the target is a polypeptide or peptide in which at least one terminus, preferably the C-terminus, is at least partially exposed. The target can be associated with a biological state such as a disease or disorder in a plant or animal (e.g., a mammal) as well as the presence of a pathogen. For example, a target can be a protein that is differentially expressed in certain cancer cells. In another example, targets such as creatine kinase, troponin I and troponin T are associated with myocardial infarction. In a further example, a target can be a protein of a pathogen such as a virus, bacterium, fungus, or single-celled organism. In some embodiments, the target can be a protein found in the exosporium of *Bacillus anthracis*, such as protein BclA. In other embodiments, the target can be one or more proteins making up a toxin such as botulinum neurotoxins of various serotypes (including heavy and light chains, as described for example in Singh, Nat. Struct. Biol., 2000, 7:617-9, and references therein), tetanus neurotoxin, or anthrax toxin (including lethal factor, protective antigen and edema factor, as reviewed for example in Stubbs, Trends Pharmacol Sci, 2002, 23:539-41, and references therein). In yet further embodiments, the target can be a polypeptide having a C-terminal sequence of EFYA.

The term “intermediate target” refers to a target that is different from the ultimately desired target but is sufficiently similar so as to aid in preparing the desired polypeptide of the invention. For example, the intermediate target can be a peptide fragment of the desired target, where the peptide fragment contains at least the last 3, 4, 5, or 6 amino acids at the carboxyl termini of the desired target. Peptides can often be easier to manipulate than large proteins. In other embodiments, the intermediate target can be a target in which the C-terminus has about 20 to about 80 percent identity with the C-terminus of the ultimately desired target. In this way, in vitro evolution of the polypeptide containing a PDZ domain can be coaxed in the desired direction. Different intermediate targets can be used in the in vitro evolution process. For example, intermediate targets having increasing percent identity can be used in successive rounds of evolution.

As used herein, the term “pathogen” refers to any microorganism, virus or prion causing disease in humans, other animals or plants, including commercially important domesticated animals and crops. Pathogens include, for example, bacteria such as *Bacillus anthracis*, *Escherichia coli* O:157, *Yersinia pestis*, *Helicobacter pylori*, *Clostridium difficile*,

Streptococcus pneumoniae, *Staphylococcus aureus*, *Mycobacterium tuberculosis*, *Mycobacterium bovis*, *Clostridium botulinum*, *Clostridium tetani* and the like. Viral pathogens include, for example, human immunodeficiency viruses (HIV), hepatitis A, B and C viruses, (HAV, HBV, HCV), respiratory syncytial virus (RSV), poliovirus, smallpox virus, 5 CMV (cytomegalovirus), flaviviruses, papillomaviruses, coronaviruses (e.g., SARS-CoV), influenza virus, viral plant pathogens such as alfalfa mosaic virus, tobacco mosaic virus, and the like. Other microbial pathogens include parasites and fungi such as, for example, *Plasmodium falciparum* (malaria) and the fungus *Candida albicans*, respectively, and the like. Prion pathogens include transmissible spongiform encephalopathies such as bovine 10 spongiform encephalopathy (BSE), Creutzfeld-Jacob disease (CJD) and the like.

As used herein, an “enzyme” is defined as any of numerous proteins that catalyze specific chemical reactions. Examples of enzymes include β -lactamases, polymerases, proteases, endonucleases, glutathione S-transferase (GST), alkaline phosphatase, and the like. Many toxins, such as cholera toxin, botulin toxin and the like, are or comprise enzymes.

15 As used herein, a “fluorescent protein” is defined as a protein having ability to fluoresce in the visible wavelengths of the electromagnetic spectrum (i.e., from about 300 nm to about 700 nm). Examples of fluorescent proteins include the Green Fluorescent Protein (GFP) and its derivatives, as well as DsRed and other proteins and their derivatives available commercially from BD Biosciences under the trademark “Living Colors”.

20 As used herein, an “epitope” is defined as a molecular region of an antigen capable of eliciting an immune response and of combining with the specific antibody produced by such a response. Epitopes can be peptides, polynucleotides, polypeptides, polysaccharides and the like.

As used herein, the term “antibody” includes polyclonal antibodies and monoclonal 25 antibodies as well as fragments thereof. Antibodies include, but are not limited to mouse, rat, and rabbit, human, chimeric antibodies and the like. The term “antibody” also includes antibodies of all isotypes. Particular isotypes of a monoclonal antibody can be prepared either directly by selecting from the initial fusion, or prepared secondarily, from a parental hybridoma secreting a monoclonal antibody of different isotype by using the sib selection 30 technique to isolate class switch variants using the procedure described in Steplewski, et al. *Proc. Natl. Acad. Sci.*, **1985**, 82, 8653 or Spira, et al., *J. Immunol. Methods*, **1984**, 74, 307.

The invention also provides fragments of the polyclonal and monoclonal antibodies described above. These “antibody fragments” typically retain some ability to selectively bind

with its antigen or immunogen. Such antibody fragments can include, but are not limited to: Fab, Fab', F(ab')₂, Fv, and SCA. An example of a biologically active antibody fragment is a CDR region of the antibody. Methods of making these fragments are known in the art, see for example, Harlow and Lane (1988), *infra*.

5 The antibodies of this invention also can be modified to create chimeric antibodies and humanized antibodies (Oi, *et al.*, *BioTechniques*, **1986**, 4(3), 214 which is incorporated herein by reference in its entirety). Chimeric antibodies are, for example, those in which the various domains of the antibodies' heavy and light chains are coded for by DNA from more than one species.

10 The isolation of other hybridomas secreting monoclonal antibodies with the specificity of the monoclonal antibodies of the invention can also be accomplished by one of ordinary skill in the art by producing anti-idiotypic antibodies (Herlyn, *et al.*, *Science*, **1986**, 232:100, which is incorporated herein by reference in its entirety). An anti-idiotypic antibody is an antibody which recognizes unique determinants present on the monoclonal antibody
15 produced by the hybridoma of interest.

 Antibodies according to the present invention can also include genetically engineered antibody fragments. For example, molecular clones of variable domains of antibodies can be transformed into single-chain variable domains (scFv), diabodies, Fab (Barbas *et al.*, *Proc. Natl. Acad. Sci. USA*, **1992**, 9, 10164), bivalent Fab (Fab'), etc., using standard recombinant
20 DNA technology. Phage display (Smith, *Science*, **1985**, 228, 1315), ribosome display (Hanes & Pluckthun, *Proc. Natl. Acad. Sci. USA*, **1997**, 94, 4937) and mRNA display (Xu *et al.*, *Chem. Biol*, **2002**, 9, 933) can be used in vitro to select antibodies with desired affinity and/or specificity.

 Laboratory methods for producing polyclonal antibodies and monoclonal antibodies,
25 as well as deducing their corresponding nucleic acid sequences, are known in the art, see, e.g., *ANTIBODIES, A LABORATORY MANUAL* (Harlow and Lane eds. (1988)) and Sambrook *et al.* *MOLECULAR CLONING: A LABORATORY MANUAL*, 2nd edition (1989), each of which is incorporated herein by reference in its entirety. The monoclonal antibodies of the present invention can be biologically produced by introducing an antigen such as a
30 protein or a fragment thereof into an animal, e.g., a mouse or a rabbit. The antibody producing cells in the animal are isolated and fused with myeloma cells or heteromyeloma cells to produce hybrid cells or hybridomas.

As used herein, "nucleic acids" or "polynucleotides" refer to polymeric forms of nucleotides or analogs thereof, of any length. The polynucleotides can contain deoxyribonucleotides, ribonucleotides, and/or their analogs. Nucleotides can have any three-dimensional structure, and may perform any function, known or unknown. The term
5 "polynucleotide" includes, for example, single-, double-stranded and triple helical molecules, a gene or gene fragment, exons, introns, mRNA, tRNA, rRNA, ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, dsRNA, and the like.

Nucleic acid molecules further include oligonucleotides, such as antisense molecules,
10 probes, primers and the like. Oligonucleotides typically have from about 2 to about 100, 8 to about 30, or 10 to about 28 nucleotides or analogs thereof.

Nucleic acid molecules can also contain modified backbones, modified bases, and modified sugars, such as for enhancing certain desirable properties such as in vivo stability, binding affinity, etc. Modifications of nucleic acids are well known in the art and include, for
15 example, modifications described in U.S. Pat. Nos. 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111; 5,563,253; 5,571,799; 5,587,361; 5,625,050; 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,434,257;
20 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,610,289; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; 5,677,439; 5,539,082; 5,714,331; 5,719,262; 5,489,677; 5,602,240; 5,034,506; 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265;
25 5,658,873; 5,670,633; 5,700,920; 3,687,808; 4,845,205; 5,130,302; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121; 5,596,091; 5,614,617; 5,681,941; 5,750,692; 5,013,830; 5,149,797; 5,220,007; 5,256,775; 5,366,878; 5,403,711; 5,491,133; 5,565,350; 5,623,065; 5,652,355; 5,652,356; and 5,700,922, each of which is incorporated herein by reference in its entirety.

30 Isolation, preparation, and manipulation of nucleic acids, is well known in the art and is well described in Sambrook, et al., *supra*.

The present invention also relates to "vectors" which include the isolated DNA molecules of the present invention, "host cells" which are genetically engineered with the

recombinant vectors, or which are otherwise engineered to produce the polypeptides of the invention, and the production of evolved PDZ domains, or derivatives thereof, by recombinant techniques.

5 The polynucleotides may be joined to a vector containing a selectable marker for propagation in a host. Generally, a plasmid vector is introduced in a precipitate, such as a calcium phosphate precipitate, or in a complex with a charged lipid. If the vector is a virus, it may be packaged in vitro using an appropriate packaging cell line and then transduced into host cells.

10 In one embodiment, the DNA of the invention is operatively associated with an appropriate heterologous regulatory element (e.g., promoter or enhancer), such as, the phage lambda PL promoter, the E. coli lac, trp, and tac promoters, the SV40 early and late promoters and promoters of retroviral LTRs, to name a few. Other suitable promoters will be known to the skilled artisan.

15 In embodiments in which vectors contain expression constructs, these constructs will further contain sites for transcription initiation, termination and, in the transcribed region, a ribosome binding site for translation. The coding portion of the mature transcripts expressed by the constructs will preferably include a translation initiating at the beginning and a termination codon (UAA, UGA or UAG) appropriately positioned at the end of the polypeptide to be translated.

20 As indicated, the expression vectors will preferably include at least one selectable marker. Such markers include dihydrofolate reductase or neomycin resistance for eukaryotic cell culture and tetracycline or ampicillin resistance genes for culturing in E. coli and other bacteria. Representative examples of appropriate hosts include, but are not limited to, bacterial cells, such as E. coli, Streptomyces and Salmonella typhimurium cells; fungal cells, 25 such as yeast cells; insect cells such as Drosophila S2 and Spodoptera Sf9 cells; animal cells such as CHO, COS and Bowes melanoma cells; and plant cells. Appropriate culture mediums and conditions for the above-described host cells are known in the art.

30 Among vectors preferred for use in bacteria include pQE70, pQE60 and pQE9, available from Qiagen; pBS vectors, Phagescript vectors, Bluescript vectors, pNH8A, pNH16a, pNH18A, pNH46A, available from Stratagene; and ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 available from Pharmacia. Among preferred eukaryotic vectors are pWLNEO, pSV2CAT, pOG44, pXT1 and pSG available from Stratagene; and pSVK3,

pBPV, pMSG and pSVL available from Pharmacia. Other suitable vectors will be readily apparent to the skilled artisan.

Selection of appropriate vectors and promoters for expression in a host cell is a well known procedure and the requisite techniques for expression vector construction, introduction of the vector into the host and expression in the host are routine skills in the art.

The present invention also relates to host cells containing the vector constructs discussed herein, and additionally encompasses host cells containing nucleotide sequences of the invention that are operably associated with one or more heterologous control regions (e.g., promoter and/or enhancer) using techniques known of in the art. The host cell can be a higher eukaryotic cell, such as a mammalian cell (e.g., a human derived cell), or a lower eukaryotic cell, such as a yeast cell, or the host cell can be a prokaryotic cell, such as a bacterial cell. The host strain may be chosen which modulates the expression of the inserted gene sequences, or modifies and processes the gene product in the specific fashion desired. Expression from certain promoters can be elevated in the presence of certain inducers; thus expression of the genetically engineered polypeptide may be controlled. Furthermore, different host cells have characteristics and specific mechanisms for the translational and post-translational processing and modification (e.g., phosphorylation, cleavage) of proteins.

Appropriate cell lines can be chosen to ensure the desired modifications and processing of the foreign protein expressed.

Introduction of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-dextran mediated transfection, cationic lipid-mediated transfection, electroporation, transduction, infection or other methods. Such methods are described in many standard laboratory manuals, such as Sambrook et al., *supra*.

As used herein, the phrase "optimization" is intended to mean any process whereby a DNA sequence encoding a translation product (polypeptide or protein) is changed to improve the expression level of this protein without altering its amino acid sequence. For instance, gene optimization can be achieved by computational methods (e.g., Fuglsang, Protein Expr Purif. 2003, 31:247-9). An alternative method of gene optimization amounts to a specialized application of directed evolution described by Stemmer et al., Gene, 1993, 123:1-7. Example expression systems include bacteria (e.g., *E. coli*) and yeast.

As used herein, the term "cell-free selection" or "cell-free screening assay" is defined as any affinity selection method which does not involve the direct use of living cells. Examples of cell-free selections include ribosome display (Hanes & Pluckthun, *Proc. Natl.*

Acad. Sci. USA, **1997**, *94*, 4937) and mRNA display (Xu *et al.*, *Chem. Biol.*, **2002**, *9*, 933). Phage display, which requires the transformation of DNA into cells in order to create selectable libraries, does not constitute an example of cell-free selection.

5 *Methods of Preparing Polypeptides*

The present invention further provides methods of preparing a polypeptide using in vitro evolution techniques. For example, a polypeptide containing a PDZ domain can be prepared by creating a library of polypeptides from one or more parent polypeptides also containing a PDZ domain. One or more polypeptides having binding affinity for a desired
10 target can then be identified from the library. In some embodiments, the identified polypeptides can be used to create a further library from which another polypeptide can be identified, potentially having even greater affinity for the selected target. Identification of library members that bind to the desired target can be carried out by any suitable methods. In some embodiments, polypeptides can be identified in a cell-free screening assay, such as
15 phage display.

In further embodiments, the present invention provides a method of preparing a polypeptide containing a PDZ domain by forming a library polypeptides from one or more parent polypeptides comprising a PDZ domain; selecting a first selected polypeptide from the library, where the first selected polypeptide has binding affinity to an intermediate target.
20 The intermediate target can have, for example, 20% to 80% sequence identity in the last 5 amino acids with the last 5 amino acids of the desired target. A further library of polypeptides can then be created from the first selected polypeptide and the process can be repeated until a library yields a polypeptide capable of binding with the desired target. The intermediate target can act as an evolutionary guide for the evolving polypeptide, and can be particularly
25 useful when the C-terminal sequence of the target is substantially different from the C-terminal sequence of the natural binder to the parent polypeptide. One or more intermediate targets can be used, and different intermediate targets can be used for each iteration.

Therapeutic and Prophylactic Methods

30 Methods of treatment according to the present invention can include both prophylaxis and therapy. Prophylaxis or therapy can be accomplished by administration to a patient of therapeutic agents such as polypeptides containing PDZ domains prepared, for example, by the directed evolution methods described herein. In some embodiments, methods of treatment

include administration of a polypeptide of the invention. In other embodiments, methods of treatment include administration of a peptide which can be bound by a polypeptide of the invention. The therapeutic agent can be administered at a single time point or multiple time points to a single or multiple sites. Administration can also be nearly simultaneous to multiple sites. Patients or subjects include mammals, such as human, bovine, equine, canine, feline, porcine, and ovine animals. The subject is preferably a human.

A disease or disorder, such as a viral infection, cancer, allergy, or other pathological condition associated with a target, can be diagnosed using criteria generally accepted in the art, including, for example, the presence of a malignant tumor or elevated white blood cell count. Therapeutic agents can be administered either prior to or following surgical removal of primary tumors and/or treatment such as administration of radiotherapy or conventional chemotherapeutic drugs. In further embodiments, therapeutic agents such as polypeptide of the invention can also be administered prior to infection by an infectious agent such as a virus, bacteria, or other pathogen.

Within certain embodiments, therapy can be immunotherapy, which can be active immunotherapy in which treatment relies on the in vivo stimulation of the endogenous host immune system (e.g., stimulation of endogenous effector cells) to react against tumors or infected cells with the administration of binding proteins prepared according to the methods described herein. Within other embodiments, immunotherapy can be passive immunotherapy, in which treatment involves the delivery of agents with, for example, immune reactivity (such as evolved PDZ domains fused to an Fc domain or conjugated to an antibody or antibody fragment) that can directly or indirectly mediate antitumor, anti-inflammatory, or other effects and do not necessarily depend on an intact host immune system. Examples of effector cells include T cells, T lymphocytes (such as CD8⁺ cytotoxic T lymphocytes and CD4⁺ T-helper tumor-infiltrating lymphocytes), killer cells (such as Natural Killer cells and lymphokine-activated killer cells), B cells and antigen-presenting cells (such as dendritic cells and macrophages).

The therapeutic agents prepared according to the methods described herein can be combined with a pharmaceutically acceptable carrier to produce a pharmaceutical composition. As used herein, "pharmaceutically acceptable carrier" includes any material which, when combined with an active ingredient, allows the ingredient to retain biological activity and is non-reactive with the subject's immune system. Examples include, but are not limited to, any of the standard pharmaceutical carriers such as a phosphate buffered saline

solution, water, emulsions such as oil/water emulsion, and various types of wetting agents. Preferred diluents for aerosol or parenteral administration are phosphate buffered saline or normal (0.9%) saline. Compositions comprising such carriers are formulated by well known conventional methods (see, for example, *Remington's Pharmaceutical Sciences*, Chapter 43, 14th Ed., Mack Publishing Co, Easton Pa. 18042, USA).

Therapeutic and Prophylactic Compositions and Uses

Much like antibodies and antibody fragments, polypeptides containing PDZ domains and their derivatives can be useful in the treatment of numerous disorders including, for example, cancer, inflammatory disorders, such as adult respiratory distress syndrome (ARDS), hypovolemic shock, ulcerative colitis, rheumatoid arthritis, and others, as shown in Table 1 which provides a list of diseases and molecular targets addressed by therapeutic antibodies.

Table 1. Monoclonal antibody-based therapeutics
(*Nature Biotechnology*, 2003, 21, 868).

Product	Company	Initial indication	Year approved
Bexxar (tositumomab; radiolabelled monoclonal antibody directed against CD20, produced in a mammalian cell line.)	Corixa/GlaxoSmith Kline	Treatment of CD20 positive follicular non-Hodgkin lymphoma	2003 (US)
Xolair (Omalizumab; rIgG1k Mab that binds IgE, produced in CHO cells)	Genentech	Asthma	2003 (US)
Humira (adalimumab; r human Mab (antiTNF) created using phage display technology and produced in a mammalian cell line)	Abbott Laboratories	Rheumatoid arthritis	2002 (US)
Zevalin (Ibritumomab Tiuxetan; murine Mab produced in a CHO cell line, targeted against the CD20 antigen. A radiotherapy agent.)	IDEC Pharmaceuticals	Non-Hodgkin lymphoma	2002 (US)
Mabcampath (EU) or Campath (US)	Millennium & ILEX (EU); Berlex,	Chronic lymphocytic leukemia	2001 (EU, US)

(alemtuzumab; a humanized monoclonal antibody directed against CD52 surface antigen of B-lymphocytes.)	ILEX & Millennium Pharmaceuticals (US)		
Mylotarg (gemtuzumab zogamicin; a humanized antibody-toxic antibiotic conjugate targeted against CD33 antigen found on leukemic blast cells.)	Wyeth	Acute myeloid leukemia	2000 (US)
Herceptin (trastuzumab, humanized antibody directed against human epidermal growth factor receptor 2 (HER2))	Genentech (US); Roche (EU)	Treatment of metastatic breast cancer if tumor overexpresses HER2 protein	1998 (US), 2000 (EU)
Remicade (infliximab, chimeric mAb directed against TNF-alpha)	Centocor	Treatment of Crohn disease	1998 (US), 1999 (EU)
Synagis (palivizumab, humanized mAb directed against an epitope on the surface of respiratory syncytial virus.)	MedImmune (US); Abbott (EU)	Prophylaxis of lower respiratory disease caused by syncytial virus in pediatric patients	1998 (US), 1999 (EU)
Zenapax (daclizumab, humanized mAb directed against the alpha-chain of the IL-2 receptor)	Hoffmann-La Roche	Prevention of acute kidney transplant rejection	1997 (US), 1999 (EU)
Humaspect (Votumumab, human mAb directed against cytokeratin tumor-associated antigen)	Organon Teknika	Detection of carcinoma of the colon or rectum	1998 (EU)
Mabthera (Rituximab, chimeric mAb directed against CD20 surface antigen of B lymphocytes. See also Rituxan.)	Hoffmann-La Roche	Non-Hodgkin lymphoma	1998 (EU)
Simulect (basiliximab, chimeric mAb directed against the alpha-chain of the IL-2 receptor)	Novartis	Prophylaxis of acute organ rejection in allogeneic renal transplantation	1998 (EU)
LeukoScan (Sulesomab, murine mAb fragment (Fab) directed against NCA 90, a surface granulocyte nonspecific cross-reacting antigen.)	Immunomedics	Diagnostic imaging for infection/inflammation in bone of patients with osteomyelitis	1997 (EU)
Rituxan (rituximab chimeric mAb directed	Genentech/IDEC Pharmaceuticals	Non-Hodgkin lymphoma	1997 (US)

against CD20 antigen found on the surface of B lymphocytes)			
Verluma (Nofetumomab murine mAb fragments (Fab) directed against carcinoma-associated antigen.)	Boehringer Ingelheim/NeoRx	Detection of small-cell lung cancer	1996 (US)
Tecnemab KI (murine mAb fragments (Fab/Fab2 mix) directed against HMW-MAA)	Sorin	Diagnosis of cutaneous melanoma lesions	1996 (EU)
ProstaScint (capromab-pentetate, murine mAb directed against the tumor surface antigen PSMA)	Cytogen	Detection/staging/ follow-up of prostate adenocarcinoma	1996 (US)
MyoScint (imiciromab-pentetate, murine mAb fragment directed against human cardiac myosin)	Centocor	Myocardial infarction imaging agent	1996 (US)
CEA-scan (arcitumomab, murine mAb fragment (Fab), directed against human carcinoembryonic antigen, CEA)	Immunomedics	Detection of recurrent/metastatic colorectal cancer	1996 (US, EU)
Indimacis 125 (Igovomab, murine mAb fragment (Fab2) directed against the tumor-associated antigen CA 125)	CIS Bio	Diagnosis of ovarian adenocarcinoma	1996 (EU)
ReoPro (abciximab, Fab fragments derived from a chimeric mAb, directed against the platelet surface receptor GPIIb/IIIa)	Centocor	Prevention of blood clots	1994 (US)
OncoScint CR/OV (satumomab pendetide, murine mAb directed against TAG-72, a tumor-associated glycoprotein)	Cytogen	Detection/staging/follow-up of colorectal and ovarian cancers	1992 (US)
Orthoclone OKT3 (Muromomab CD3, murine mAb directed against the T-lymphocyte surface antigen CD3)	Ortho Biotech	Reversal of acute kidney transplant rejection	1986 (US)

Therapeutic formulations of polypeptides of the invention or derivatives thereof can be prepared for storage by mixing the polypeptide or derivative thereof having the desired

degree of purity with optional pharmaceutically acceptable carriers, excipients, or stabilizers (see, e.g., *Remington's Pharmaceutical Sciences*, *supra*), in the form of lyophilized cake or aqueous solutions. Acceptable carriers, excipients or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and
5 other organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrans; chelating agents such as EDTA; sugar
10 alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as Tween, Pluronics or polyethylene glycol (PEG).

Polypeptides of the invention or derivatives thereof for *in vivo* administration are preferably sterile. This can be readily accomplished by filtration through sterile filtration membranes, prior to or following lyophilization and reconstitution. The polypeptides of the
15 invention or derivatives thereof ordinarily will be stored in lyophilized form or in solution.

Therapeutic polypeptide compositions generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle.

The route of polypeptide administration can be carried out in accord with known
20 methods, e.g., inhalation, injection or infusion by intravenous, intraperitoneal, intracerebral, intramuscular, intraocular, intraarterial, or intralesional routes, by enema or suppository, or by sustained release systems as noted below. The polypeptide or its derivative is given systemically or at a site of inflammation.

Suitable examples of sustained-release preparations include semipermeable polymer
25 matrices in the form of shaped articles, e.g. films, or microcapsules. Sustained release matrices include polyesters, hydrogels, polylactides (U.S. Pat. No. 3,773,919 and EP 58,481), copolymers of L-glutamic acid and gamma ethyl-L-glutamate (Sidman et al., *Biopolymers*, **1983**, 22, 547), poly (2-hydroxyethyl-methacrylate) (Langer et al., *J. Biomed. Mater. Res.*, **1981**, 15, 167 and Langer, *Chem. Tech.*, **1982**, 12, 98), ethylene vinyl acetate (Langer et al.,
30 *supra*) or poly-D-(-)-3-hydroxybutyric acid (EP 133,988). Sustained-release compositions also include liposomally entrapped evolved PDZ domain or derivative thereof. Liposomes containing an evolved PDZ domain or derivative thereof can be prepared by methods known per se: DE 3,218,121; Epstein et al., *Proc. Natl. Acad. Sci. U.S.A.*, **1985**, 82, 3688; Hwang et

al., *Proc. Natl. Acad. Sci. U.S.A.*, 1980, 77, 4030; EP 52,322; EP 36,676; EP 88,046; EP 143,949; EP 142,641; U.S. Pat. Nos. 4,485,045 and 4,544,545; and EP 102,324. Ordinarily the liposomes are of the small (about 200-800 Angstroms) unilamellar type in which the lipid content is greater than about 30 mole percent cholesterol, the selected proportion being
5 adjusted for the most efficacious therapy.

An "effective amount" of a polypeptide of the invention to be employed therapeutically will depend, for example, upon the therapeutic objectives, the route of administration, and the condition of the patient. Accordingly, it may be necessary for the therapist to titer the dosage and modify the route of administration as required to obtain the
10 optimal therapeutic effect. Typically, the clinician will administer the polypeptide until a dosage is reached that achieves the desired effect. The progress of this therapy is easily monitored by conventional assays.

In the treatment and prevention of a disease or disorder, the polypeptide composition can be formulated, dosed, and administered in a fashion consistent with good medical
15 practice. Factors for consideration in this context include the particular disorder being treated, the particular mammal being treated, the clinical condition of the individual patient, the cause of the disorder, the site of delivery of the polypeptide, the particular type of polypeptide, the method of administration, the scheduling of administration, and other factors known to medical practitioners. The "therapeutically effective amount" of polypeptide to be
20 administered can be governed by such considerations, and is the minimum amount necessary to prevent, ameliorate, or treat the inflammatory disorder. Such amount is preferably below the amount that is toxic to the host or renders the host significantly more susceptible to infections.

As a general proposition, the initial pharmaceutically effective amount of the
25 polypeptide administered parenterally per dose can be in the range of about 0.1 to 50 mg/kg of patient body weight per day, with the typical initial range of polypeptide used being 0.3 to 20 mg/kg/day, more preferably 0.3 to 15 mg/kg/day. As noted above, however, these suggested amounts of polypeptide are subject to therapeutic discretion.

The polypeptide of the invention need not be, but is optionally formulated with one or
30 more agents currently used to prevent or treat the disease or disorder in question. For example, in rheumatoid arthritis, a polypeptide can be given in conjunction with a glucocorticosteroid, or for cancer, a polypeptide can be given in conjunction with a

chemotherapeutic. The polypeptide can also be formulated with one or more other polypeptides of the invention to provide a therapeutic “cocktail.”

Methods of Detection

5 The invention further provides a method for detecting a disease, disease-causing pathogen or disorder such as cancer in a sample, comprising contacting the sample with a polypeptide containing a PDZ domain that binds to a target in the sample, where the target is associated with the disease or disorder. The target can be, for example, a nucleic acid or protein encoded thereby. The target can be a substance, such as a peptide or protein that is
10 produced directly by a pathogen, including toxins and the like. The sample can be an environmental sample, or a tissue from a mammal, such as human, bovine, equine, canine, feline, porcine, and ovine tissue. In some embodiments, the tissue is human. The tissue can comprise a tumor specimen, cerebrospinal fluid, or other suitable specimen such a tissue likely to contain the target of interest. In one embodiment, the method comprises use of an
15 ELISA type assay that employs an evolved PDZ domain or derivative thereof by the methods described herein to detect the presence of target in a specimen. This method can also be used to monitor target levels in a tissue sample of a patient. For example, the suitability of a therapeutic regimen for initial or continued treatment can be determined by monitoring target levels according to this method.

20 The invention further provides a method for detecting a disease, disease-causing pathogen or disease such as cancer in a patient by administering a polypeptide of the invention to the patient and detecting binding of the polypeptide in the patient. In some embodiments, the administered polypeptide further contains a reporter group, such as a radioactive moiety, chelated heavy metal, or other imaging agent to facilitate detection of
25 binding of the polypeptide in the patient. Binding of polypeptide in the patient can be observed as localization of the polypeptide in certain tissues containing the desired target. For example, a polypeptide of the invention that is capable of specifically binding to a cancer marker such as a polypeptide differentially expressed from certain cancer cells can reveal the presence of a tumor or diseased tissue by detection of localization of the polypeptide.
30 Methods for scanning a patient, such as a human patient, are well known in the art and include radiography, MRI, and related techniques.

 The practice of the present invention employs, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques),

microbiology, cell biology, biochemistry and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. These methods are described in the following publications. See, e.g., Sambrook, et al., *Molecular Cloning: A Laboratory Manual*, 2nd edition (1989); *Current Protocols in Molecular Biology* (F. M. Ausubel et al. eds. (1987)); the series *Methods in Enzymology* (Academic Press, Inc.); *PCR: A Practical Approach* (M. MacPherson et al. IRL Press at Oxford University Press (1991)); *PCR 2: A Practical Approach* (M. J. MacPherson et al., eds. (1995)); *Antibodies, A Laboratory Manual* (Harlow and Lane eds. (1988)); *Animal Cell Culture* (R. I. Freshney ed. (1987)); and *Phage Display: A Laboratory Manual* (C.F. Barbas III et al., (2001)), each of which is incorporated
5
10 herein by reference in its entirety.

Methods of purification

The present invention further provides a method of purifying a protein comprising contacting said protein with an immobilized polypeptide containing a PDZ domain, wherein
15 the immobilized polypeptide has binding affinity for the protein.

The following examples are presented to illustrate the present invention and to assist one of ordinary skill in making and using the same. The examples are not intended in any way to otherwise limit the scope of the invention.
20

EXAMPLES**EXAMPLE 1. Synthesis of human CASK PDZ domain gene optimized for expression in *Escherichia coli* and *Saccharomyces cerevisiae*.**

A gene fragment, hCASK-PDZopt, having the sequence shown in SEQ ID NO: 1 is
5 obtained from a commercial supplier (GENEART, Germany). This hCASK-PDZopt codes
for the PDZ domain of the human hCASK gene (GenBank accession number AF032119)
product. The sequence of this PDZ domain is provided in SEQ ID NO: 2. The gene
fragment of SEQ ID NO:1 is designed for optimal expression in both *Escherichia coli* and
Saccharomyces cerevisiae. The gene fragment is cloned into vector pCR-Script Amp
10 (Stratagene, LaJolla, CA) using *KpnI* and *SacI* restriction sites and transformed into *E. coli*
XL10-Gold (Stratagene). DNA sequencing using standard labeled-dideoxy terminator
chemistry and an Applied BioSystems instrument is carried out to verify the sequence of the
cloned synthetic gene.

15 EXAMPLE 2. Construction and expression of translational fusion of GST and human hCASK PDZ synthetic gene.

The synthetic gene of SEQ ID NO:1, hCASK-PDZopt, is sub-cloned from the pCR-
Script vector of Example 1 into plasmid pGEX-2TK (Amersham Biosciences) using *EcoRI*
and *BamHI* restriction sites. This yields plasmid pGEX-hCASK-PDZopt, comprising a
20 translational fusion whose open reading frame includes the GST gene fused to the synthetic
PDZ domain gene fragment. DNA sequencing is carried out according to standard methods
to confirm that the DNA sequence of the subclone codes for the protein provided in SEQ ID
NO: 3, namely GST fused to the hCASK PDZ domain.

pGEX-hCASK-PDZopt DNA is transformed into *E. coli* strain JM109 for expression
25 of the GST-hCASK PDZ fusion protein and purification via affinity chromatography using
glutathione sepharose affinity medium (Amersham Biosciences). Purified protein is
visualized by coomassie blue-stained SDS-PAGE.

Function of the GST moiety is confirmed by incubating 1 µg of the fusion protein
with 1-chloro-2,4-dinitrobenzene (CDNB, provided by Amersham Biosciences) and reduced
30 glutathione, as described by the manufacturer, in 0.1M potassium phosphate buffer, pH 6.5,
and monitoring absorbance at 340 nm. Increase of absorbance of > 0.02 OD/min in the first 5
minutes of the reaction is expected for functional GST-CASK fusion protein.

EXAMPLE 3. Construction and expression of translational fusion of alkaline phosphatase gene and human hCASK PDZ synthetic gene.

The synthetic gene of SEQ ID NO: 1, hCASK-PDZopt, is fused with the alkaline phosphatase gene of *Escherichia coli* (*phoA*) via overlap PCR, a well-known technique
5 (Horton et al., 1990, Biotechniques, 8, 528), to yield a gene encoding the polypeptide shown in SEQ ID NO: 4. The 5' primer used to amplify the hCASK-PDZopt gene fragment encodes a sequence of amino acids corresponding to the signal sequence shown in SEQ ID NO: 4. The outer-most primers are designed to provide convenient restriction sites (*NcoI* and *HindIII*) for cloning the gene coding into plasmid pQE-60 (Qiagen, www.qiagen.com)
10 digested with *NcoI* and *HindIII* restriction sites. This yields plasmid pQE-hCASK-PDZopt-alkphos, comprising a translational fusion whose open reading frame includes the alkaline phosphatase gene of *Escherichia coli* fused to the synthetic PDZ domain gene fragment. DNA sequencing is carried out according to standard methods to confirm that the DNA sequence of the subclone codes for the protein provided in SEQ ID NO: 4.

15 pQE-hCASK-PDZopt-alkphos DNA is transformed into *E. coli* strain JM109 for expression of the alkaline phosphatase-hCASK PDZ fusion protein and purification via affinity chromatography using streptavidin-sepharose (Amersham Biosciences) to which the N-terminal-biotinylated peptide ligand of hCASK PDZ (obtained from any of many custom peptide suppliers such as Invitrogen) is bound. Purified protein is visualized by coomassie
20 blue-stained SDS-PAGE. Function of the alkaline phosphatase moiety is confirmed by incubating 1µg of the fusion protein with para-nitrophenol phosphate colorimetric substrate (cat. No. A3469, Sigma, St-Louis, MO) monitoring absorbance at 405 nm. Increase of absorbance above background is expected. In contrast, a control such as GST-CASK fusion protein incubated in the same conditions is expected to yield a change of absorbance similar
25 to background (i.e., substrate alone).

EXAMPLE 4. Construction and expression of translational fusion of immunoglobulin Fc gene and synthetic human hCASK PDZ synthetic gene.

The synthetic gene of SEQ ID NO: 1, hCASK-PDZopt, is fused with a human
30 immunoglobulin Fc gene fragment by overlap PCR, a well-known technique (Horton et al., 1990, Biotechniques, 8, 528), to yield a gene encoding the polypeptide shown in SEQ ID NO: 6. The 5' primer used to amplify the hCASK-PDZopt gene fragment encodes a sequence of amino acids corresponding to the signal sequence of human light chain immunoglobulin

shown in SEQ ID NO: 6. The outer-most primers are designed to provide convenient restriction sites for cloning the gene coding into plasmid pCDNA3.1(+)*myc*/*his*/*LacZ* (Qiagen, www.qiagen.com) digested with *Hind*III and *Pme*I restriction sites. This yields plasmid pCDNA-hCASK-PDZopt-Fc, comprising a translational fusion whose open reading frame includes the human immunoglobulin Fc gene fragment fused to the synthetic PDZ domain gene fragment. DNA sequencing is carried out according to standard methods to confirm that the DNA sequence of the subclone codes for the protein provided in SEQ ID NO: 6.

Plasmid pCDNA-hCASK-PDZopt-Fc, linearized away from the hCASK PDZ-Fc fusion gene using a unique restriction site, is transfected according to well-known procedures (Sambrook & Russell, 2001, Molecular cloning: a laboratory manual) into mammalian cell line NS0 approvable for the production of recombinant immunoglobulins for therapeutic use. Stable transfectants are screened for clones producing useful amounts of the fusion protein. Fusion protein produced in this fashion can be isolated from the culture medium and purified using standard antibody affinity purification resins such as Protein G sepharose (Amersham Biosciences). The protein can be assayed for biological activity or ability to bind a ligand.

EXAMPLE 5.1. Use of GST-hCASK PDZ variant fusion as an affinity reagent (Western blotting and ELISA.)

Variants of purified GST-hCASK PDZ fusion protein such as those described in examples 10 and 13, (see also example 14) are used as an affinity reagent to detect proteins which bind to the PDZ moiety of the fusion protein. In this example, the affinity matured GST-hCASK PDZ fusion of example 12 is used to detect syndecan-2. The GST moiety acts as an epitope tag, or reporter domain. To detect syndecan-2 in human brain tissues, the brain tissues are homogenized, suspended in SDS-PAGE reducing sample buffer (Fermentas) and boiled for 3 minutes. The samples are resolved by SDS-PAGE and western transfer is carried out to blot the separated proteins onto a membrane according to standard methods. The blot is then blocked with I-block (Applied Biosystems) according to instructions from the manufacturer and probed using affinity-matured GST-hCASK PDZ fusion protein. The membrane is washed and probed with a secondary antibody specific to GST and labeled with horseradish peroxidase (Amersham Biosystems). Chemiluminescence is used to detect the secondary antibody according to chemiluminescence kit manufacturer protocols (Vector Labs).

To detect the protein without electrophoretic separation, an ELISA is carried out. In this example, affinity-matured GST-hCASK PDZ is immobilized on the bottom of the wells of an ELISA plate (1µg/well). The wells of the plate are then blocked with I-block (Applied Biosystems), washed with buffer, and a brain homogenate sample is added to the well. The samples are allowed to incubate for 2 hours at room temperature. The plate is washed, and the presence of syndecan-2 is determined by using a secondary antibody specific to syndecan-2 (Zymed laboratories, www.zymed.com), and, following incubation and wash, a tertiary goat anti-rabbit antibody labeled with horseradish peroxidase (VWR, www.vwr.com). Chemiluminescence is used to detect the tertiary antibody – and indirectly, the target syndecan-2 –according to chemiluminescence kit manufacturer protocols (Vector Labs).

EXAMPLE 5.2. Use of alkaline phosphatase-hCASK PDZ fusion as an affinity reagent (Western blotting and ELISA.)

Purified alkaline phosphatase-CASK PDZ fusion protein of example 3, or variants of this protein such as described in examples 10 and 13, (see also example 14) is used as an affinity reagent to detect proteins that bind to the PDZ moiety of the fusion protein. As explained in example 5.1, the protein to be detected is bound to a solid support, either via western transfer, or via direct or indirect adsorption to one or more wells of a multi-well assay plate (ELISA plate). Instead of using an anti-GST antibody for detection, as was done in example 5.1, binding of the PDZ domain or variants thereof is detected via the alkaline phosphatase reporter domain fused to the PDZ domain. Detection is carried out using Vector labs' DuoLuX chemiluminescent/fluorescent substrate for alkaline phosphatase according to the manufacturer's recommendations.

EXAMPLE 6. Error-prone PCR mutagenesis of hCASK PDZ gene.

The synthetic gene fragment of SEQ ID NO: 1, hCASK-PDZopt, is excised from vector pCR-Script with *Sfi*I and *Not*I restriction enzymes and ligated into the pre-digested pCANTAB5E phagemid (Amersham Biosciences) using Fast-Link DNA ligation kit (Epicentre Technologies, Madison, WI). The ligated DNA is transformed into electroporation-competent *E. coli* XL1-Blue. A phage ELISA is performed, confirming that recombinant phage displaying CASK PDZ domain bind specifically to its cognate peptide ligand (QKAPTKEFYA). DNA sequencing of the pCANTAB-hCASK-PDZopt construct is also carried out, ensuring that the sequence of the construct is as expected.

The hCASK-PDZopt gene is mutated by error-prone PCR (Leung et al., 1989, Technique, 1: 11-15), yielding a mutant library containing over 10^6 unique mutants. Primers pCAN5' (CATGATTACGCCAAGCTTTGG) and pCAN3' (CGATCTAAAGTTTTGTCGTC) are used to amplify the PDZ gene under mutagenic conditions. To prepare the library, the mutated PCR product is digested with *Sfi*I and *Not*I and ligated into the pCANTAB5E phagemid vector (Amersham Biosciences) using Fast-Link DNA ligation kit (Epicentre Technologies). The ligated DNA is then transformed into electroporation-competent *E. coli* XL1-Blue. Several frozen stocks are made from cultures of the mutant libraries and stored at -80°C for future use. The complexity of the library is determined by counting the number of colonies obtained after plating an aliquot of the freshly transformed cells on agar-containing LB medium and ampicillin. A complexity of about 10^6 unique clones is expected. The observed mutation rate corresponds approximately to between 1 and 3 amino acid substitutions per gene as determined by DNA sequencing of randomly picked clones.

15

EXAMPLE 7. Random combinatorial mutagenesis of hCASK PDZ gene.

Amino acids likely to affect the specificity of the hCASK-PDZ gene product were identified by inspection of the crystal structure of hCASK PDZ, PDB number 1KWA (Daniels et al., Nat Struct Biol. 1998, 5, 317-25), using freely available Viewerlite 4.2 software (www.accelrys.com). Residues M501, I503, L505, Q553, L556 and R557 were identified as being in close contact with the C-terminal residue of the peptide recognized by hCASK PDZ (numbering scheme is according to Daniels et al., 1998, 5, 317-325). These residues were selected for randomization via combinatorial mutagenesis to create a single library in which any of the 20 amino acids can be found at these mutated positions in individual variants.

25

Codons corresponding to amino acids M501, I503, L505, Q553, L556 and R557 of the hCASK PDZ gene are mutated by amplification of the gene using primers pCAN5' (CATGATTACGCCAAGCTTTGG) and NNK1B (CAATGATTCAATTCATTCATTTMNNGGTMNNACCMNNTGGTTCATCGGTATTT TTTTG), NNK2A (AAAATGAATGAATTGAATCATTG) and NNK2B (GGTAATAGAACCACGCATTTMNNMNNCATTMTMNNCAATTGTTCAACGGTTTG ATTGG), as well as NNK3A (GAAATGCGTGGTTCTATTACC), and pCAN3' (CGATCTAAAGTTTTGTCGTC) to generate three overlapping PCR products: codons

30

M501, I503, L505 are randomized in the first product, and codons encoding residues Q553, L556 and R557 are randomized in the second. Overlap PCR is carried out (Horton et al., 1990, Biotechniques, 8, 528) using the three purified PCR products to produce a pool of mutant genes of the following degenerate sequence:

5 “CATGATTACGCCAAGCTTTGGAGCCTTTTTTTTGGAGATTTTCAACGTGAAAAAA
TTATTATTCGCAATTCCTTTAGTTGTTCTTTCTATGCGGCCAGCCGGCCGGATC
CGGTATGGATATGGAAAATGTTACCCGTGTTTCGTTTAGTTCAATTTCAAAAAAAT
ACCGATGAACCAN**NKGGTNNK**ACC**NNK**AAAATGAATGAATTGAATCATTGTATT
GTTGCCCCGATTATGCATGGTGGTATGATTCATCGTCAAGGTACTTTGCATGTTGG
10 TGATGAAATTCGTGAAATTAATGGTATTTCTGTTGCCAATCAAACCGTTGAACAA
TTG**NNK**AAAATG**NNKNNK**GAAATGCGTGGTTCTATTACCTTTAAATTTGTTCCAT
CTTATCGTACCCAATCTTCTTCTGGAATTCATGCGGCCGCAGGTGCGCCGGTGCC
GTATCCGGATCCGCTGGAACCGCGTGCCGCATAGACTGTTGAAAGTTG”, where N
signifies any of the four nucleotides, A, C, G or T, and K signifies either of the two
15 nucleotides G or T. The underlined sequence codes for the PDZ domain and the bold codons
(**NNK**) are degenerate. To prepare a library of combinatorial mutants, the mutated PCR
product is digested with *Sfi*I and *Not*I and ligated into the pCANTAB5E phagemid vector
(Amersham Biosciences) using Fast-Link DNA ligation kit (Epicentre Technologies). The
ligated DNA is then transformed into electroporation-competent *E. coli* XL1-Blue. Several
20 frozen stocks are made from cultures of the mutant libraries and stored at -80°C for future
use. The complexity of the library (over 10⁷ unique clones) is determined by counting the
number of colonies obtained after plating an aliquot of the freshly transformed cells on agar-
containing LB medium and ampicillin. Presence of the expected mutations is verified by
DNA sequencing of randomly picked clones.

25

EXAMPLE 9. Target Set Mutagenesis of hCASK PDZ gene.

Amino acids likely to determine the specificity of the hCASK-PDZ gene product were identified by inspection of the crystal structure of hCASK PDZ, PDB number 1KWA (Daniels et al., Nat Struct Biol. 1998, 5, 317-25), using freely available Viewerlite 4.2
30 software (www.accelrys.com). Residues M501, I503, L505, Q553, L556 and R557 were
identified as being in close contact with the C-terminal residue of the peptide recognized by
hCASK PDZ. These residues were selected for Target Set Mutagenesis (Goldman and

Youvan, Biotechnology (N Y), 1992, 10, 1557-61) to create a single library in which a subset, or target set, of the 20 amino acids is encoded at each mutated codon.

Each target set corresponds to the amino acids encountered at homologous positions in an alignment of related PDZ domains. The sequence alignment, shown in FIGURE 1, was
 5 obtained by using the amino acid sequence of the hCASK PDZ domain as a query in a BLAST search of the non-redundant protein sequence database (<http://www.ncbi.nlm.nih.gov/>). Six different target sets were determined based on this alignment: for residue 501, amino acids M or L; for residue 503, amino acids I, L, V, or A; for residue 505, amino acids V, I, L, or F; for residue 553, amino acids I or Q; for residue
 10 556, amino acids L, I or M; for residue 557, amino acids R, K, or S. For each of these target sets, a degenerate codon is computed using the program Cyberdope (Kairos Scientific, San Diego, CA): for residue 501, the degenerate codon MTG yields amino acids M or L; for residue 503, the degenerate codon VYT yields amino acids I, L, T, P, V, or A; for residue 505, the degenerate codon NTT yields amino acids I, L, V, or F; for residue 553, the
 15 degenerate codon MWK yields amino acids I, K, L, M, N, H or Q; for residue 556, the degenerate codon MTK yields amino acids I, L, or M; for residue 557, the degenerate codon ARK yields amino acids R, K, S or N. (The encoded amino acids do not always match exactly the target set due to the structure of the genetic code.) Where A = adenosine, C = cytidine, G = guanosine, T = thymidine, B = C or G or T, D = A or G or T, H = A or C or T,
 20 K = G or T, M = A or C, N = A or C or G or T, R = A or G, S = C or G, V = A or C or G, W = A or T, Y = C or T, according to the IUPAC code. Oligonucleotides are then synthesized encoding the degenerate codons.

Codons corresponding to amino acids M501, I503, L505, Q553, L556 and R557 of the hCASK PDZ gene are mutated by amplification of the gene using oligonucleotide primers
 25 pCAN5' (CATGATTACGCCAAGCTTTGG) and TSM1B (CAATGATTCAATTCATTCATTTTAANGGTARBACCCAKTGGTTCATCGGTATTTT TTTG), TSM2A (AAAATGAATGAATTGAATCATTG) and TSM2B (GGTAATAGAACCACGCATTTCTMYTMAKCATTTTMMWKCAATTGTTCAACGGTTT GATTGG), as well as TSM3A (GAAATGCGTGGTTCTATTACC), and pCAN3'
 30 (CGATCTAAAGTTTTGTCGTC) to generate three overlapping PCR products: codons M501, I503, L505 are mutated in the first product, and codons encoding residues Q553, L556 and R557 are mutated in the second. Overlap PCR is carried out (Horton et al., 1990, Biotechniques, 8, 528) using the three purified PCR products to produce a pool of mutant

genes of the following degenerate sequence:

- “CATGATTACGCCAAGCTTTGGAGCCTTTTTTTTGGAGATTTTCAACGTGAAAAAA
 TTATTATTCGCAATTCCTTTAGTTGTTTCCTTTCTATGCGGCCAGCCGGCCGGATC
 CGGTATGGATATGGAAAATGTTACCCGTGTTTCGTTTAGTTCAATTTCAAAAAAAT
 5 ACCGATGAACCAMTGGGT**VYTACCNT**TAAAAATGAATGAATTGAATCATTGTATT
GTTGCCCCGATTATGCATGGTGGTATGATTCATCGTCAAGGTACTTTGCATGTTGG
TGATGAAATTCGTGAAATTAATGGTATTTCTGTTGCCAATCAAACCGTTGAACAA
TTGMW**K**AAAAATG**MTKARK**GAAATGCGTGGTTCTATTACCTTTAAAATTGTTCCA
TCTTATCGTACCCAATCTTCTTCTGGAATTCATGCGGCCCGCAGGTGCGCCGGTGC
 10 CGTATCCGGATCCGCTGGAACCGCGTGCCGCATAGACTGTTGAAAGTTG”, where
 A = adenosine, C = cytidine, G = guanosine, T = thymidine, B = C or G or T, D = A or G or
 T, H = A or C or T, K = G or T, M = A or C, N = A or C or G or T, R = A or G, S = C or G,
 V = A or C or G, W = A or T, Y = C or T, according to the IUPAC code. The underlined
 sequence codes for the PDZ domain and the bold codons are degenerate. To prepare a library
 15 of combinatorial mutants, the mutated PCR product is digested with *Sfi*I and *Not*I and ligated
 into the pCANTAB5E phagemid vector (Amersham Biosciences) using Fast-Link DNA
 ligation kit (Epicentre Technologies). The ligated DNA is then transformed into
 electroporation-competent *E. coli* XL1-Blue. Several frozen stocks are made from cultures of
 the mutant libraries and stored at -80°C for future use. The complexity of the library (over
 20 10⁷ unique clones) is determined by counting the number of colonies obtained after plating an
 aliquot of the freshly transformed cells on agar-containing LB medium and ampicillin.
 Presence of the expected mutations is verified by DNA sequencing of randomly picked
 clones.

25 **EXAMPLE 10. Selection of hCASK PDZ variant recognizing *Bacillus anthracis* protein BclA.**

Affinity selection

- In this example, the error-prone PCR library of example 6, above, is selected for
 mutants that are capable of recognizing a peptide of the sequence SASIIIEKVA
 30 corresponding to the C-terminus of protein BclA which is found in the exosporium of
Bacillus anthracis spores. Phage displaying the hCASK-PDZ library variants are prepared
 according to standard methods (e.g., Barbas et al., 2001) from frozen stocks of the library.
 The library is carried through 5 rounds of panning using N-terminal-biotinylated peptide

SASIIIEKVA bound to streptavidin coated onto polystyrene wells of multiwell plates (Nunc). Phage binding specifically to SASIIIEKVA peptide-coated wells are allowed to infect *E. coli* XL1-Blue simply by adding cells to the well and incubating them for 15 minutes. The input phage titer (number of phage added to a well) and output phage (phage removed from well) from each round are determined. The ratio of output phage to input phage for each round of panning typically shows a clear trend of phage amplification after Round 3 or 4, suggesting selection of mutants specific for the target peptide SASIIIEKVA.

Mutant Screening

A phage ELISA is performed on about 20 randomly chosen clones from each of panning rounds 3, 4 and 5 to verify that the selection is successfully amplifying mutants binding specifically to peptide SASIIIEKVA. Log-phase XL1-Blue cultures are infected with the mutant phage output from each panning round. An aliquot of this infected culture is then plated onto agar-containing medium and allowed to grow 16 hours. Multiple clones are picked, grown in 96-well polypropylene culture plates, infected with helper phage, and the resulting culture supernatant used in a phage ELISA. Several clones from round 5 produce a strong binding signal to peptide SASIIIEKVA and are chosen for further characterization.

Mutant characterization

Phage are purified by PEG/NaCl precipitation from three mutants as well as wildtype controls and tested against biotinylated peptides HRRSARYLDTVL, QKAPTKEFYA, and SASIIIEKVA by phage ELISA. Each mutant shows dramatically improved ELISA signal for peptide SASIIIEKVA compared to wildtype hCASK-PDZ, and only weak binding to control peptides HRRSARYLDTVL and QKAPTKEFYA. These mutants are therefore capable of specifically binding to peptide SASIIIEKVA.

Confirmation of BclA binding

Characterized mutants showing binding to peptide SASIIIEKVA are then further characterized; their ability to bind *Bacillus anthracis* BclA is determined. Phagemid DNA of the selected mutants is purified per standard methods, digested with *EcoRI* and *BamHI* and the resulting variant hCASK-PDZ gene fragment is ligated to pGEX-2TK DNA digested with the same restriction enzymes. The resulting ligated DNA is transformed into *E. coli* strain JM109 to yield clones containing plasmid pGEX-PDZ-variant. These clones are grown to an

OD600 of 0.5 to 1.0 and induced using 1mM IPTG for 5 hours at 22°C. The induced cells are pelleted by centrifugation and lysed using a French press. PDZ variant-GST fusion protein is purified from the lysate via affinity chromatography using glutathione sepharose affinity medium. Purified PDZ variant-GST fusion protein is then tested for its ability to bind
5 *Bacillus anthracis* BclA by using the PDZ variant-GST fusion as an affinity reagent, as described in example 5.1 above, wherein protein BclA is present on a western blot or coated onto the wells of a multi-well plate in an ELISA format.

EXAMPLE 11. Recursive Ensemble Mutagenesis of hCASK PDZ gene.

10 The random combinatorial library described in example 7 is subjected to affinity selection, as described in example 10. A few different PDZ variants capable of binding target peptide SASIIIEKVA are isolated. The aim of the present example is to isolate further variants having improved binding affinity towards the target peptide. The DNA sequences of the few different PDZ variants are determined and used to design a new combinatorial library
15 wherein a bias is introduced towards the expression of those amino acids observed at the randomized positions of the isolated PDZ variants (Delagrave et al., 1993, Protein Eng, 6: 327-31). To design this new library, the amino acids encountered in the isolated PDZ variants are compiled for each mutated position (i.e., residues M501, I503, L505, Q553, L556 and R557). The list of amino acids is entered into a computer program called CyberDope,
20 available from Kairos Scientific (San Diego, CA). The program is instructed by the operator to use the group probability option (P_G) and the NNK (NN[G/T]) codon option. The program then provides a nucleotide mixture (degenerate codon) which encodes all of the amino acids encountered at the mutated position of interest. A list of amino acids is entered for each mutated position (i.e., residues M501, I503, L505, Q553, L556 and R557) resulting in a
25 degenerate codon for each position.

Oligonucleotides comprising the degenerate codons provided by the computer program are synthesized by a custom oligonucleotide manufacturer (Integrated DNA Technologies, Coralville, IA). Using these oligos, a new combinatorial library is synthesized by PCR. The new library is then selected by affinity panning, as described in example 10.
30 New variants having improved affinity are selected from this library. The selected further PDZ variants are then tested for improved ability to bind the target protein, as described in example 13, using BIAcore measurements of affinity.

EXAMPLE 12. Affinity maturation of wildtype PDZ.

The affinity of the wildtype PDZ of hCASK towards its target protein syndecan-2 can be improved by a process of *in vitro* affinity maturation. This is done by first mutating, via error-prone PCR, the gene of hCASK PDZ, thereby creating a library as described in example 6. Secondly, affinity selection is applied to select for variants having improved affinity as described in example 10, except that biotinylated peptide QKAPTKEFYA, corresponding to the C-terminus of syndecan-2, is used instead of biotinylated peptide SASIIIEKVA. Individual selected variants are grown, their DNA purified, and the PDZ gene fragments of the variants are sub-cloned, as described in examples 2 and 10. The resulting GST-PDZ variant fusions are purified, per example 10, and tested to compare their affinities towards the target protein syndecan-2. The cytoplasmic domain of syndecan-2 is attached to the microfluidic chip of a BIAcore instrument (BIAcore, Piscataway, NJ) and GST-PDZ variants are analyzed using the instrument to calculate their binding affinities. Variants showing improved affinity compared to the parent hCASK PDZ demonstrate the effectiveness of this procedure. Such improved variants can be useful as research reagents, diagnostics or therapeutics.

EXAMPLE 13. Affinity maturation of PDZ variants.

The affinity of a PDZ variant isolated in example 10 above, towards its target protein, BclA, can be improved by a process of *in vitro* affinity maturation. This is done by first mutating, via error-prone PCR, the gene of the PDZ variant, thereby creating a library as described in example 6. Secondly, affinity selection is applied to select for variants having improved affinity, as described in example 10. Individual variants are grown, their DNA purified, and the PDZ gene fragments of the variants are sub-cloned, as described in examples 2 and 10. The resulting GST-PDZ variant fusions are purified, per example 10, and tested to compare their affinities towards the target protein. The target protein is attached to the microfluidic chip of a BIAcore instrument (BIAcore, Piscataway, NJ) and GST-PDZ variants are analyzed using the instrument, to calculate their binding affinities. Variants showing improved affinity compared to the parent PDZ variant demonstrate the effectiveness of this procedure. Such improved variants can be useful as research reagents, diagnostics or therapeutics.

Example 14. Construction of PDZ variant fusion proteins and their use as affinity reagents.

Any of the evolved PDZ variants isolated in examples 13, 12, 11, or 10 can be made into translational fusions essentially as described for wildtype PDZ domain in examples 2, 3 and 4. Any of the resulting fusion proteins can be used as reagents for detection of the peptides or proteins which these PDZ variants have been evolved to bind, essentially as described in examples 5.1 and 5.2.

Example 15. Affinity purification using evolved PDZ domain.

An evolved PDZ domain binding to a target protein is isolated according to any of the above examples. The evolved PDZ domain is purified and attached to beaded agarose affinity medium using the Aminolink Plus immobilization kit (Pierce, www.piercenet.com). The target protein is then isolated from a complex mixture by using the immobilized evolved PDZ domain according to standard affinity chromatography procedures.

Example 16: Sequences**SEQ ID NO: 1. DNA sequence of hCASK-PDZopt.**

TTTTTATGCGGCCAGCCGGCCGGATCCGGTATGGATATGGAAAATGTTACCCGT
GTTCTGTTTAGTTCAATTTCAAAAAAATACCGATGAACCAATGGGTATTACCTTGA
AAATGAATGAATTGAATCATTGTATTGTTGCCCGTATTATGCATGGTGGTATGAT
TCATCGTCAAGGTACTTTGCATGTTGGTGATGAAATTCGTGAAATTAATGGTATT
TCTGTTGCCAATCAAACCGTTGAACAATTGCAAAAAAATGTTGCGTGAAATGCGTG
GTTCTATTACCTTTAAAATTGTTCCATCTTATCGTACCCAATCTTCTTCTGGAATTC
ATGCGGCCGCTGGTGCTCCAGT

SEQ ID NO: 2. Amino acid sequence encoded by the underlined DNA sequence of hCASK-PDZopt gene fragment shown in SEQ ID NO: 1.

GMDMENVTRVRLVQFQKNTDEPMGITLKMNELNHCIVARIMHGGMIHRQGLHVG
DEIREINGISVANQTVEQLQKMLREMRGSITFKIVPSYRTQSSS

SEQ ID NO: 3. Amino acid sequence of hCASK-PDZ-GST fusion.

MSPILGYWKIKGLVQPTRLLEYLEEKYEEHLYERDEGDKWRNKKFELGLEFPNLPY
YIDGDVKLTQSMARIYIADKHNMLGGCPKERAEISMLEGAVLDIRYGVSRIAYSKDF

ETLKVDFLSKLPEMLKMFEDRLCHKTYLNGDHVTHPDFMLYDALDVVLYMDPMCL
 DAFPKLVCFFKKRIEAIQIDKYLKSSKYIAWPLQGWQATFGGGDHPPKSDLVPRGSR
 RASVGSGMDMENVTRVRLVQFQKNTDEPMGITLKMNELNHCIVARIMHGGMIHRQGT
LHVGDEIREINGISVANQTVEQLQKMLREMGRSITFKIVPSYRTQSSSGIHRD

- 5 (The hCASK-PDZ amino acid sequence is shown in italics.)

SEQ ID NO: 4. Amino acid sequence of hCASK-PDZ-alkaline phosphatase fusion.

MSIQHFRVALIPFFAAFCPLPVFAGMDMENVTRVRLVQFQKNTDEPMGITLKMNELNHCIV
VARIMHGGMIHRQGT*LHVGDEIREINGISVANQTVEQLQKMLREMGRSITFKIVPSYRTQS*
 10 *SSRTPEMPLQGTAVDGGGGSMHASLEVLENRAAQGDITAPGGARRLTGDQTAALRD*
SLSDKPAKNIILLIGDGMGDSEITAARNYAEGAGGFFKGIDALPLTGQYTHYALNKKT
GKPDYVTDASAATAWSTGVKTYNGALGVDIHEKDHPITLEMAKAAGLATGNVST
AELQDATPAALVAHVTSRKCYPGATSEKCPGNALEKGGKGSITEQLLNARADVTL
GGGAKTFAETATAGEWQGKTLREQAQARGYQLVSDAASLNSVTEANQQKPLLGLF
 15 *ADGNMPVRWLGPATYHGNDKPAVTCPTNPQRNDSVPTLAQMTDKAIELLSKNEK*
GFFLQVEGASIDKQDHAANPCGQIGETVDLDEAVQRALEFAKKEGNTLVIVTADHA
HASQIVAPDTKAPGLTQALNTKDGAVMVMSYGNSEEDSQEHTGSQLRIAAYGPHAA
NNVGLTDQTDLFYTMKAALGLK

- (the hCASK-PDZ domain sequence is italicized, the leader sequence (*E. coli* β -lactamase
 20 TEM) is underlined, and the remainder of the sequence corresponds to alkaline phosphatase
 of *E. coli*.)

SEQ ID NO: 6. Amino acid sequence of hCASK-PDZ-Fc fusion protein.

MRAPAQIFGFLLLLFPGTRCGMDMENVTRVRLVQFQKNTDEPMGITLKMNELNHCIV
 25 ARIMHGGMIHRQGT*LHVGDEIREINGISVANQTVEQLQKMLREMGRSITFKIVPSYRTQSSSE*
PKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVDVSHEDPEVK
FNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKDYGKCKVSNKAL
PAPMQKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPRHIAVEWESNG
QPENNYKTTTPVLDSGDSFFLYSKLTVDKSRWQQGNVFCFVMSHEALHNHYTQKSL
 30 *SLSPGK*

(Underlined sequence is the signal sequence of light chain human immunoglobulin.
 Italicized sequence is the hCASK-PDZ domain, and the remainder of the sequence
 corresponds to the human IgG1 Fc domain sequence.)

- 35 **SEQ ID NO: 7. Amino acid sequence of polyhistidine tagged and secreted hCASK-PDZ.**

MSIQHFRVALIPFFAAFCCLPVFAGMDMENVTRVRLVQFQKNTDEPMGITLKMNELNHCI
VARIMHGGMIHRQGTTLHVGDEIREINGISVANQTVEQLQKMLREMRGSITFKIVPSYRTQS
SSHHHHHH

(The hCASK-PDZ domain sequence is italicized, the leader sequence (E. coli β -lactamase
5 TEM) is underlined, and the C-terminal six residues correspond to the polyhistidine tag.)

SEQ ID NO: 8. Amino acid sequence of secreted hCASK-PDZ.

MSIQHFRVALIPFFAAFCCLPVFAGMDMENVTRVRLVQFQKNTDEPMGITLKMNELNHCI
VARIMHGGMIHRQGTTLHVGDEIREINGISVANQTVEQLQKMLREMRGSITFKIVPSYRTQS
10 SS

(The hCASK-PDZ domain sequence is italicized, the leader sequence (E. coli β -lactamase
TEM) is underlined.)

All publications and patent applications cited in this specification are herein
15 incorporated by reference as if each individual publication or patent application were
specifically and individually indicated to be incorporated by reference. Although the
foregoing invention has been described in some detail by way of illustration and example for
purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in
the art in light of the teachings of this invention that certain changes and modifications may
20 be made thereto without departing from the spirit or scope of the appended claims.

What is claimed is:

1. A polypeptide comprising a PDZ domain, wherein said PDZ domain binds to a target produced by a pathogen or disease state.
2. The polypeptide of claim 1 wherein said pathogen is viral.
3. The polypeptide of claim 1 wherein said pathogen is fungal.
4. The polypeptide of claim 1 wherein said pathogen is bacterial.
5. The polypeptide of claim 1 wherein said pathogen is of the genus *Bacillus*.
6. The polypeptide of claim 1 wherein said pathogen is *Bacillus anthracis* or *Clostridium botulinum*.
7. The polypeptide of claim 1 wherein said disease is cancer.
8. The polypeptide of claim 1 wherein said target is a polypeptide.
9. The polypeptide of claim 1 wherein said target is found in the exosporium of *Bacillus anthracis*.
10. The polypeptide of claim 1 wherein said target is protein BclA of *Bacillus anthracis* or a fragment thereof.
11. The polypeptide of claim 1 wherein said target is a polypeptide having a C-terminal sequence of EFYA.
12. The polypeptide of claim 1 wherein said PDZ domain is evolved.
13. The polypeptide of claim 1 wherein said PDZ domain is evolved from the PDZ domain of protein hCASK.
14. The polypeptide of claim 1 wherein said PDZ domain is evolved from SEQ ID NO: 2.
15. The polypeptide of claim 1 wherein said PDZ domain is a variant of the PDZ domain of protein hCASK.
16. The polypeptide of claim 1 wherein said PDZ domain is a variant of SEQ ID NO: 2.
17. The polypeptide of claim 1 further comprising a reporter group.
18. The polypeptide of claim 17 wherein said reporter group comprises an enzyme.
19. The polypeptide of claim 17 wherein said reporter group comprises a fluorescent protein.
20. The polypeptide of claim 17 wherein said reporter group comprises an epitope.

21. The polypeptide of claim 1 further comprising an effector domain.
22. The polypeptide of claim 21 wherein said effector domain comprises an antibody fragment.
23. The polypeptide of claim 21 wherein said effector domain comprises a toxin.
24. The polypeptide of claim 21 wherein said effector domain comprises a protein transduction domain.
25. The polypeptide of claim 1 further comprising a radioactive isotope.
26. The polypeptide of claim 1 wherein said polypeptide is isolated.
27. A polynucleotide encoding the polypeptide of claim 1.
28. A vector comprising the polynucleotide of claim 27.
29. A host cell comprising the polynucleotide of claim 27.
30. An antibody that binds to said polypeptide of claim 1.
31. A method of detecting the presence of a pathogen or disease in a patient comprising:
 - a) administering a polypeptide of claim 1 to said patient; and
 - b) detecting binding of said polypeptide in said patient.
32. A method of detecting the presence of a pathogen or disease in a sample comprising:
 - a) contacting a polypeptide of claim 1 with said sample; and
 - b) detecting binding of said polypeptide to said sample.
33. The method of claim 32 wherein said polypeptide further comprises a reporter group.
34. The method of claim 32 wherein said detecting is carried out by Western blot or ELISA.
35. The method of claim 32 wherein said sample comprises a bacterial pathogen.
36. The method of claim 32 wherein said sample comprises *Bacillus anthracis*, *Clostridium botulinum* or their toxins.
37. The method of claim 32 wherein said sample comprises a viral pathogen.
38. A method of preparing a polypeptide comprising a PDZ domain, wherein said PDZ domain binds to a target produced by a pathogen or disease state, comprising:
 - a) creating a library of polypeptides from one or more parent polypeptides comprising a PDZ domain;
 - b) identifying one or more polypeptides from said library having binding affinity for said target.

39. The method of claim 38 wherein said library of polypeptides is created by combinatorial mutagenesis
40. The method of claim 38 wherein said library of polypeptides is created by error-prone PCR.
41. The method of claim 38 wherein said one or more parent polypeptides is optimized for expression in a desired expression system.
42. The method of claim 38 wherein said expression system is bacterial.
43. The method of claim 38 wherein said expression system is yeast.
44. The method of claim 38 wherein said identifying is carried out in a cell-free screening assay.
45. The method of claim 38 wherein said identifying is carried out by phage display.
46. A polypeptide comprising a PDZ domain and an effector domain.
47. The polypeptide of claim 46 wherein said effector domain comprises a protein transduction domain, an Fc domain, or serum albumin.
48. A polypeptide comprising a PDZ domain, wherein said PDZ domain binds to a target, wherein said polypeptide is prepared by:
 - a) creating a library of polypeptides from a parent polypeptide comprising a PDZ domain having SEQ ID NO: 2;
 - b) identifying said polypeptide having binding affinity for said target from said library.
49. The polypeptide of claim 48 wherein said target is associated with a pathogen or disease.
50. The polypeptide of claim 49 wherein said disease is cancer.
51. The polypeptide of claim 49 wherein said pathogen is bacterial.
52. A polypeptide comprising a PDZ domain, wherein said PDZ domain binds to a target, wherein said polypeptide is prepared by recursive ensemble mutagenesis.
53. The polypeptide of claim 52 wherein the target is target produced by a pathogen or disease state.
54. The polypeptide of claim 53 wherein said disease is cancer.
55. The polypeptide of claim 53 wherein said pathogen is bacterial.
56. A library of polypeptides prepared from a parent polypeptide comprising a PDZ domain, said parent polypeptide comprising SEQ ID NO: 2.

57. A method of treating a disease associated with a pathogen, comprising administering to a patient infected with or likely to become infected with said pathogen a therapeutically effective amount of a polypeptide comprising a PDZ domain capable of binding to a target associated with said pathogen.
58. The method of claim 57 wherein said pathogen is *Bacillus anthracis*.
59. The method of claim 57 wherein said target comprises a toxin produced by *Bacillus anthracis*.
60. The method of claim 57 wherein said pathogen is *Clostridium botulinum*.
61. The method of claim 57 wherein said target comprises a toxin produced by *Clostridium botulinum*.
62. The method of claim 57 wherein said pathogen is *Clostridium tetani*.
63. The method of claim 57 wherein said target comprises a toxin produced by *Clostridium tetani*.
64. A method of preparing a polypeptide comprising a PDZ domain, wherein said PDZ domain binds to a polypeptide target associated with a pathogen, comprising:
- a) forming a library of polypeptides from one or more parent polypeptides comprising a PDZ domain;
 - b) selecting a first polypeptide from said library, said first polypeptide having binding affinity to an intermediate target having 20% to 80% sequence identity in the last 5 amino acids with the last 5 amino acids of said target;
 - c) creating a further library of polypeptides from the first polypeptide of step b);
 - d) repeating steps b) and c) until a polypeptide that binds with said target is identified.
65. A method of purifying a protein comprising contacting said protein with an immobilized polypeptide comprising a PDZ domain, wherein said immobilized polypeptide has binding affinity for said protein.

ABSTRACT

The present invention provides polypeptides that contain a PDZ domain and are useful in the detection of pathogens. The polypeptides of the invention are also useful in the diagnosis, treatment, and prevention of diseases. Also provided are methods of preparing polypeptides of the invention.

FIGURE 1

<u>1_1256</u>	1	RLVQFQKNTDEPMGITLKMNELNHCIVARIMHGGMIHRQGTILHVGDEIREINGISVANQT	60
<u>22203757</u>	489	548
<u>2736069</u>	72	.E.A.E..QS..L.V...L.DKQR.S....L.....S..E....A....K.....	131
<u>1346574</u>	72	.E.A.E..QS..L.V...L.DKQR.S....L.....S..E....A....K.....	131
<u>28277667</u>	129	.MIGIR.KAG..L.V.FR.DK-GDLVI...L...L.D...L.....I.K.V..KD.GTNP	187
<u>12856535</u>	138	KIIRLV..S-..L.A.I.KD.QTGA.....R..AAD.S.LI.....L..V...P.EDKR	197
<u>31222171</u>	147	KM.GIRR.P...L.L.VEVD.H.QLV....IA....D...L..P..V.L.V..VP.T--.	204
<hr/>			
<u>1_1256</u>	61	VEQLQKMLREMRGSITFKIVPSYR	84
<u>22203757</u>	549	572
<u>29378343</u>	576	.DV..S..K.A....IL.....L.	599
<u>105150</u>	130	.D....AMK.TK.M.SL.VI.N	151
<u>33303973</u>	130	.D....AMK.TK.M.SL.VI.N	151
<u>12856535</u>	198	P.EII.I.SQSK.A.....I..TK	221
<u>29437038</u>	198	P.EII.I.SQSK.A.....I..TK	221
<u>31222171</u>	205	P.E..GEISVAKE.V.L..G..	226

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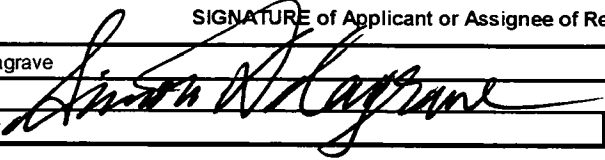
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